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ROOT TUBERCLES OF LEGUMINOSAE.

BY ERWIN F. SMITH.

Among those who have contributed to our knowledge of this subject are Beyerinck, Frank, Ward, Hellriegel, Prazmowski, Nobbe, Schlossing, Laurent and Windogradski. The question of the symbiotic relationship of the bacilli, which are certainly present in the tubercles, has received rather more attention from these investigators than have the bacteria themselves. The latter are the subject of an interesting paper, "Die Bakterien in den Wurzelknöllchen der Leguminoseen," by Mr. Gonnermann in *Landw. Jahrb.*, XXIII (1894), Heft., 4, 5, pp. 649–671. The first part of the work was done at the Agr. Exp. Sta. in Rostock, and the rest in the Hygienic Laboratory at Danzig, and the internal evidence of the paper indicates a careful, competent man. The one question which the author at first set out to solve by means of purely bacteriological methods was, What bacterium causes the tubercles? Pure cultures were made from the bacteria occurring inside the tubercles and their behavior first studied on ordinary culture media—gelatine, agar, potato, bouillon, etc. Subsequently, lupine gelatine was used, and proved very suitable, the germs growing in it about equally well, whether slightly acid, slightly alkaline or neutral. The colonies which appeared on this gelatine were then inoculated into various media, from the plates to stick cultures, from these to potato, from the latter to agar, from agar into hanging drops, from these to plates once more, and so on, to insure purity and absolute certainty of the final results. To obtain material for making infections, uninjured tubercles were washed in ordinary water and the earth rubbed away with a tooth-brush, then washed several times in distilled water, and finally put for several minutes into 1-500 solution mercuric chloride. They were then thoroughly washed 3–4 times with sterile water, placed under a bell-jar on a glass plate previously heated to 150° C., cut open with a flamed knife, and crushed out in a little sterile water, which was then used for cover glass preparations and for the inoculation of culture media. All staining fluids and all culture media were examined for the presence of germs before they were used, and before commencing this investigation the author made a preliminary one of the air of his laboratory to determine what germs were present and might be expected to appear in some of the cultures. The microscope used was a Leitz, which was provided with apochromatic lenses, giving a very clear, sharp field, up even to 2,250 diameters. The root sections were made in the Pathological-anatomical Institute of Dr. Thierfelder, and mostly by Dr. Thierfelder, himself. Several hundred plants were investigated, including *Pisum sativum, Lupinus angustifolius, albus, luteus, Lathyrus tuberosus, Vicia faba, cracca, Phaseolus vulgaris* and *Trifolium incarnatum*, and more than 300 permanent preparations were made. The investigations finally covered the following subjects: (a) Pure cultures; (b) Search for the organisms in the soil; (c) Germination of sterilized seeds in sterile sand and subsequent infection of the plants. Cover-glass preparations, made from great numbers of cleaned, sterilized tubercles of *Lupinus albus* and *angustifolius* showed the well-known Y-shaped bodies and gelatine plate cultures gave two sorts of colonies, both bacilli. Cleaned and superficially sterilized roots were then wrapped in freshly sterilized cotton, put in turn into sterile netting, and finally covered by a fine-meshed sterile wire netting, buried in sterile sand and watered with sterile water. After eight days the plants were pulled up. Many of the tubercles were ruptured and the enveloping cotton was stained brown and swarming with pure growths of the bacteria. The sand was also contaminated. From this infected cotton, and also frequently from the sand, cultures were made into gelatine, bouillon, etc., and from these, plate cultures. The author cannot agree with Frank that the Y-form consists of broken down mycoplasma, for, upon being placed in hanging drops, these Y's break up into motile bacilli and their compound nature can also be demonstrated by proper staining. Beyerinck, Prazmowski and Frank speak of one organism designated variously as *Bacillus radicicola, Bacterium radicicola,* and *Rhizobium leguminosarum.* Gonnermann thinks that there are several germs capable of causing these galls. He calls his organisms *Bacillus tuberigenus,* 1, 2, 3, etc., having isolated no no less than seven varieties, not including two *micrococci.* All of these are characterized, but not as fully as the present state of bacteriology requires. Beyerinck's *B. radicicola* was not found. Soil examinations were begun at Rostock. Earth was scattered on gelatine plates, and soil from lupine fields was washed with sterile water and cultures made from this. By these methods four of the kinds already isolated from the tubercles made their appearance and were cultivated out and their identity established. The most abundant organism in the Rostock fields was *Bacillus fluorescens non liquefaciens,* then followed *B. tuberigenus,* No. 3. This is a motile organism, 0.3 by 0.6μ, united in 2's or more, bright red-brown on potato, yellow-brown or brownish and fine granular on gelatine plates, and able to liquify gelatine rapidly. Winter examinations of earth were made for spores. In soil taken from Rostock, in February, not a living bacterium could be found, but there were numerous spores. This soil was shaken up with sterile water, and the coarsest parts allowed to settle as sediment I. The cloudy fluid was poured off into a sterile test-tube and allowed to settle for a minute to get sediment II. Sediments III and IV were obtained in the same manner, the latter consisting of the finest silt. Cover-glass preparations were made from each sediment and stained with gentian violet for the identification of bacteria, while for spores a corresponding series was dry-heated to 150°C., and then exposed for an hour to boiling carbol fuchsin, washed in alcohol, and afterward, in some cases, faintly stained with methyl blue. Finally, plate cultures were made from each sediment. Sediment I contained numerous bacilli, 4–9, by 0.5–0.6μ, each bearing 2–6 spores. No bacteria free from spores could be found, but plate cultures gave many colonies. No such large bacilli were found in the earth in summer. In sediment II, spore-bearing bacilli were few, but plate cultures yielded many colonies, thus showing the presence of spores. In sediment III, dead Y-forms first appeared. These stained faintly with ordinary reagents, but distinct round bodies appeared in their interior when they were subjected to the spore stain. In sediment IV, no bacilli were found, but there were small stained bodies which might well be spores, and plate cultures gave numerous colonies. The plate cultures from these sediments yielded unquestionable *B. tuberigenus* 1, 2, 3. The remaining forms appeared to be ordinary soil bacteria, and were not followed further.

From the results of these cultures and the examination of a great many cover glass preparations, the author thinks it is established that the tubercle organisms pass the winter in the earth in the form of spores. Sand cultures and infections were made at Rostock and again at Danzig, the following method being employed. The sand was spread out in an oven and heated for five hours at 150° C. It was then put into 3-litre pots, previously washed many times in boiling distilled water, then several times in 1-500 solution of mercuric chloride, and finally in sterile water. The pots were then covered tightly with sterile cotton and set aside. Subsequently they were infected with organisms directly from the tubercles and also with pure cultures of the same. In the Rostock experiments the pots were watered with Frank's salt mixture and in the others they received only sterile water, bacteria being added from time to time to each watering fluid. The seeds planted in these pots were first soaked ten minutes in 1-500 sol. mercuric chloride and then washed thoroughly in sterile water. The plants grew slowly, but on the whole satisfactorily. When they reached a height of 20 cm., one which had been infected directly from a tubercle was pulled and examined. The rest of the plants prospered and no more were pulled until they were in bloom. Close together on the roots of the plant first pulled there were 5 tubercles. On cutting they showed the rose red color, and the Y-forms were clearly visible on microscopic examination. Similar results had been obtained by previous investigators. More important, therefore, is the result of the infections with cultures known to be pure. Plants grown in pots infected with *B. tuberigenus* No. 3 from Rostock and others grown in pots infected with B. tuberigenus No. 5

from near Danzig developed a considerable number of tubercles in which it was very easy to demonstrate the Y-shaped bodies, and from which pure cultures of Nos. 3 and 5 were again obtained. Since these two forms behave differently on culture media, the author insists that it is no longer a question of one tubercle bacillus, but thinks that there are at least two and probably more, the form varying with the locality. Water cultures were carried on along with the sand cultures, using peas and lupines, but with negative results. Some of the roots decayed and none developed tubercles. Hellriegel first advanced the hypothesis (1886) that the bacteria in these tubercles are capable of taking nitrogen from the air and turning it over to the host plant. This striking hypothesis at once came into favor and was accepted as proved by many writers on agricultural topics. Frank, however, in dry material, found no increase whatever of nitrogen when his Rhizobium grew with the plants. His many experiments show that the garden bean (*Phaseolus vulgaris*) which always bears tubercles under natural conditions never becomes any richer in nitrogen than do beans grown in sterile soil and free from tubercles. This certainly looks more like parasitism than symbiosis. Other experiments made by Frank show that lupines and peas can assimilate nitrogen when grown in sterile humus, and free from tubercles and bacteria. Consequently leguminous plants are able to store nitrogen and enrich the soil without the action of bacteria, and it is not settled how the nitrogen is taken up by the plant. Gonnermann reasoned that if the bacilli really assimilate free nitrogen and turn it over to the host plant, then when they are grown in an artificial medium the latter ought finally to become somewhat richer in nitrogen. Following out this idea, very careful experiments were made with potato broth of a known nitrogen content, but although the bacteria grew luxuriantly for 14 days there was absolutely no increase of nitrogen. The cultures were made in 12 150 cc. flasks and every 24 hours the air was changed, being passed through cotton, strong sulphuric acid, and strong potash liquor to free it from dust, microörganisms, ammonia and carbon dioxide. The analyses were made by Dr. Meyer of the Rostock Agricultural Experiment Station. Experiments by the author confirm Hellriegel's view that the tubercle bacilli are not capable of changing ammonium salts into nitrate, and the evidence is very good that these organisms are not the same as the nitrifying ferments of Windogradski. The Y-form occurs sparingly outside of the tubercles in various parts of the plant. The author also isolated *B. tuberigenus* from tubercles found on the roots of the rape plant. His general conclusions are as follows:

(1). The root tubercles of the Leguminosae are not caused by a single specific bacterium but rather by several, one in one locality, another in another locality.

(2). The Y-forms are zoogloea (Gebildkomplexe) which arise in the plant during the symbiotic or parasitic relations, and later when the tubercles rupture, they break up into the individual bacteria. These pass into the soil, form spores, and in the spring, as bacilli, once more enter the plant to again become Y-complexes during its growth.

(3). The symbiotic relations are not yet known with certainty, for *of themselves* the tubercle bacteria of the Leguminosae are not capable of rendering free nitrogen useful to the plant; much rather is the plant in condition *of its ownself* to take up and use elementary nitrogen without fungous symbiosis. The bacteria aid the plant in doing this and may contribute in part to a higher nitrogen content. Finally, it appears to be established that in spite of the presence of the bacteria the plants do not take up any excess of nitrogen. From the many sided experiments which have been made, it follows also that not merely symbiotic *but also parasitic influences* are at work, and that the function of the bacteria as well as the method of assimilation of free nitrogen is not yet known with any certainty.